Uptake study in *Juncus sp.* and *Salicornia europaea* of six pharmaceuticals by liquid chromatography quadrupole time-of-flight mass spectrometry

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Abstract

In this work, eight plants of *Juncus sp*. and ten of *Salicornia europaea* were used for an uptake assay of pharmaceuticals (flumequine, cirpofloxacin, enrofloxacin, carbamazepine, diclofenac and ibuprofen) by irrigation at three concentration levels: 10 ng mL^{-1} (low level); 700 ng mL^{-1} (medium level) and 10 µg mL^{-1} (high level). Two plants irrigated with pharmaceutical-free water were set up as controls. For each level, two plants were watered every day with 50 mL (*Juncus sp*.) and every two days with 20 mL (*Salicornia europaea*) of aqueous solutions containing all the analytes at the described concentrations. Plants irrigated at 10 µg mL^{-1} were significantly the most affected, whereas the rest of the plants remained, in general, largely displayed no apparent physiological effects throughout the 30 days (*Juncus sp*.) and 21 days (*Salicornia europaea*) assays. Leaves and stems were cut every seven days and roots were collected at the end of the assay. The samples were lyophilized, submitted to a microwave assisted extraction using 5 mL of acetonitrile:water mixture (1:1, v/v) and they were analyzed (in triplicate) in a liquid chromatography-quadrupole time of flight mass spectrometry instrument. Most of the analytes were quantified in many of the samples corresponding to the three exposure levels with the highest concentrations obtained at high exposure levels. Ibuprofen was not detected in any sample and enrofloxacin, ciprofloxacin and diclofenac were not detected in the samples from *Salicornia europaea.*

Keywords*: Juncus sp., Salicornia europaea, liquid chromatography quadrupole time-of-flight mass spectrometry, pharmaceuticals, uptake and translocation of pharmaceuticals.*

1. Introduction

Scientific researchers and government regulators are focusing attention on trace quantities of emerging pollutants in wastewater effluents and surface waters (Wilkinson et al, 2017), the major emerging contaminants are new pesticides, pharmaceuticals, personal care products, surfactants, phthalates (Gorito et al., 2017), and perfluorinated alkylated substances (PAFS) (Mengmeng et al., 2019), resulting in an increased level of concern regarding the potential environmental impact of these compounds (Wilkinson et al, 2017).

The use of treated wastewater for irrigation in many areas has become a common practice and since 1991, European Union regulations established that all its member states must treat urban wastewater before it is discharged into the environment, lakes, rivers and seas (EC Council Directive, 1991) in order to avoid the increasing rates of pollutants in ecosystems.

The increase in the consumption of pharmaceutical products has become an environmental pollution problem, since most wastewater treatment plants lack methods for the adequate pharmaceutical products elimination from wastewaters. These substances still remain present and are discharged into environmental surface waters, where the active components of the pharmaceuticals undergo biodegradation processes at different rates (Szymonik et al., 2012). The processes used in wastewater treatment plants (WWTPs) do not efficiently remove many substances, including those considered emerging contaminants like pharmaceuticals (Tauxe-Wuersch et al., 2005; Hijosa-Valesco et al., 2011; Li, 2014; Rahdar et al., 2019). The pharmaceuticals that are less susceptible to biodegradation in water can be stored in aquatic ecosystems and have been shown to exhibit toxic effects on fish and several aquatic micro-organisms (Huerta et al., 2018). Authors (Fernández-Torres et al., 2011) have found several antibiotics in marine fish samples from southern Spain. Another problem is that pharmaceuticals might infiltrate into groundwater causing contamination of drinking water, which presents a problem for human health (Szymonik et al., 2012). Plants are capable of incorporating, mainly through their roots, the compounds dissolved in the water that irrigates them such as the long-studied for years heavy metals (Arasimowicz et al., 2013; Martínez-Alcalá et al., 2017). However, less attention was paid to the accumulation of pharmaceuticals in plants until the first decade of 2000s, and the majority of studies have been mainly focused in vegetables intended for human

Abbreviations: NSAIDs: non-steroidal anti-inflamatory drugs, DCL: Diclofenac, IBU: Ibuprofen, CBZ: Carbamazepine, CPR: Ciprofloxacin, ENR: enrofloxacin, FMQ: Flumequine

consumption while very little work has been done on this topic with wild plant species (Eggen et al., 2011; Wu et al., 2013, 2014; Marsoni et al., 2014; Hurtado et al., 2016; Riemenschneider et al., 2017a, 2017b; Di Baccio et al., 2017; Montemurro et al., 2017; Martínez-Piernas et al. 2018; Picó et al., 2019).

Most common pharmaceuticals detected in surface waters include compounds such as *non-steroidal anti-inflammatory drugs (NSAIDs)* like diclofenac (DCL) or ibuprofen (IBU), which are persistent in the environment and have been found in water streams (Qureshi et al., 2019); *anti-epileptic drugs* such as carbamazepine (CBZ) (Szymonik et al., 2012) and antibiotics from several families with, in general, long-term effects on all ecosystems (Liu et al., 2018).

The presence of ciprofloxacin (CPR) in the environment has shown adverse effects on fish (Ziarrusta et al., 2018). For enrofloxacin (ENR), the studies carried out by Wang et al., 2019 revealed non acceptable levels of this substance in an aquatic environment. The toxicity of ENR on agricultural crops has also been evaluated by finding levels of ENR in water between 50-5000 μg L^{-1} producing toxic effects on both plants and animals that are able to metabolize ENR to CPR. (Migliore et al., 2003). The use of pharmaceuticals and their occurrence in the environment have received significant attention owing to antibiotic-resistant bacteria, but their presence in environmental samples near protected areas, livestock farming areas or crops should be monitored to establish effective strategies for reducing their use and evaluate their effects on the surrounding ecosystems. (Chansik et al, 2018).

The high consumption of ibuprofen increases its presence in soils and waters, and it is considered one important emerging pharmaceutical pollutant due to its generalized presence in the natural environment (Di Baccio et al., 2017, Huang et al., 2020).

Carbamazepine (CBZ) is a frequently detected pharmaceutical compound in aquatic environments, causing chronic toxicity and endocrine disruption in a variety of non-target aquatic organisms (Chen et al., 2019). Several studies have shown its presence in different environmental samples, such as water and sediments due to inefficient disposal from wastewater treatment plants or even through direct dumping of untreated wastewaters (Gros et al., 2010; Camacho-Muñoz et al., 2010, 2012; Verlicchi et al., 2012). Its presence in protected areas such as Doñana National Park, among other

protected areas, has aroused great interest (Camacho-Muñoz et al., 2013; Carmona et al., 2014; Rivera-Jaimes et al., 2018) due to its potential risk for surrounding ecosystems.

Therefore, in this work we have studied the accumulation of different active pharmaceutical substances (flumequine (FMQ), cirproloxacin, enrofloxacin, carbamazepine, diclofenac and ibuprofen) on two types of plants that grow mainly in aquatic ecosystems, seeking to assess the incidence, at laboratory scale, that discharge of waters containing pharmaceuticals would have in an aquatic ecosystem. These plants were selected because they are widespread in the ecosystems of the Mediterranean area where several protected areas are located. *Juncus sp.* grows on the banks of rivers and wetlands and *Salicornia europaea*, grows in coastal marshes and inland salt habitats, for being a halophilic plant, although it is also found in different nature reserves such as the Yellow River Nature Reserve (China) (Wang et al., 2020) or in the Sierra de Cartagena (Spain) (Ottenhof et al., 2007). Different investigations have shown the capacity of different *Salicornia* types for phytoremediation, for example the studies of (Kannan et al., 2009) show the capacity of *Salicornia brachiata* as a phytoremediation agent in the accumulation of NaCl in wastewater (Kannan et al., 2009). Or the use of *Salicornia remosissima* in the phytoremediation of various metals such as cadmium (Rathore et al., 2016). Also, some studies, have showed the power of *Juncus effusus*, as well as *Juncus inbricatus*, to remove metals and other freshwater pollutants from waste or runoff. (Bobadilla et al., 2013; Zhang et al., 2019). This survey was designed to evaluate the presence of the selected analytes in the leaves/stems and roots of these plants, with phytoremediation capability, for two purposes, on one hand, evaluating their possible use in the recovery of contaminated ecosystems and on the other hand, studying their absorption capability versus pharmaceuticals which would have implications on wild ecosystems.

2. Materials and methods

2.1. Chemicals and reagents.

A Suprapur formic acid (98-100% purity) from Merck Darmstadt (Germany) was used. LC-MS Ultra Chromasolv® acetonitrile and water were supplied from Sigma-Aldrich (Madrid, Spain). Ultrapure water from a Milli-Q Plus (Millipore, Billerica, MA, USA) was used for aqueous solutions and

dilutions. Waters (Barcelona, Spain) supplied sodium hydroxide in 2- propanol:water and Leucine Enkephalin.

Pharmaceutical standards (ENR, CPR, FMQ, DCL, CBZ and IBU) were 98% purity or higher and were purchased from Sigma–Aldrich (Madrid, Spain). Stock solutions of 100 $\mu g \cdot mL^{-1}$ in methanol were prepared for the determinations and uptake study except for CPR that was prepared diluting 2.5 mg in 5 mL ultrapure water first and volume was made up to 25 mL with methanol. Adequate aqueous dilutions from 100 $\mu g \cdot mL^{-1}$ stock solutions were prepared daily as working solutions.

2.2. Uptake study design.

To carry out the uptake studies, *Juncus sp.* plants of 30-40 cm length were purchased in a local plant nursery ensuring that way the homogeneity of the specimen during the assay. *Salicornia europaea* plants (15-25 cm length) were supplied by AGRO-ON/ Riafresh® (Faro, Portugal). The plants were left to acclimatize for two weeks, and those that seemed unhealthy were discarded before the experiment began. The plants were grown in a greenhouse in pots to guarantee the homogeneity of the substrate and to maintain conditions similar to the natural environment with humidity of 70% and a temperature between 25 and 30ºC. *Juncus sp*. plants were cultivated in a universal substrate, while those of *Salicornia europaea* as it is a halophilic plant were grown in a coconut fiber substrate.

For the experiment eight plants of *Juncus sp.* and ten of *Salicornia europaea* were used. The assay was performed at three concentration levels: low, medium and high level of exposition corresponding to 10 $ng \cdot mL^{-1}$, 700 $ng \cdot mL^{-1}$, and 10 $\mu g \cdot mL^{-1}$ respectively. Two plants per level were irrigated with 50 mL of solutions containing the six drugs at the concentrations described above for 30 days, in the case of *Juncus sp.*, and 21 days with 20 mL, for *Salicornia europaea*, therefore, the solution remained in the soil until the next water addition and the specific humidity needs for each type of plant were maintained. *Juncus sp.* plants were irrigated every day and *Salicornia europaea* every 2 days pouring the solution into the potting soil directly.

Additionally, the same irrigation pattern with pharmaceuticals-free water, was used with two plants per assay set to be used as blanks (controls).

The cuts of leaf/stems (composite sample) were performed every 7 days and the samples were processed for analysis to determine the quantity of compound uptaken. Samples from each plant were analyzed in triplicate for leaves/stems and roots. The roots were collected at the end of the assay in order to evaluate the accumulation.

2.3. Sample treatment.

The obtained samples (leaf/stem and roots) were first washed with tap water removing soil and other residues and then with deionized water to finally dry them with a paper towel. Finally, the samples were lyophilized, ground to powder and stored until extraction at -80°C.

The extraction was made using a previous optimized procedure (Barreales-Suárez et al., 2018). Briefly, 1.0 g of homogeneous lyophilized sample was accurately weighted into a PTFE microwave pressurized vessel and it was extracted with 5 mL of a mixture acetonitrilo (ACN): water (H_2O) (1:1 v/v). A 50 W (Ethos One, Millestone, Sorisole (BG), Italy) microwave power was applied for 5 min to extract the samples. The extracts were centrifuged at 6000 rpm for 15 min and 1 mL of the supernatant was diluted 1:5 (v/v) with a mixture ACN:H_2O (1:1 v/v). Finally, before their injection into the UPLC system the extracts were microfiltered through 0.22 µm PTFE syringe filters (VWR, Spain).

2.4. Liquid chromatography- mass spectrometry analysis

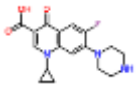
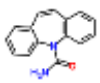
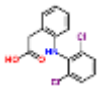
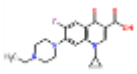
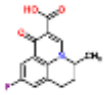
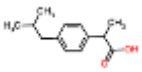
A previous optimized and validated method was used for the determinations (Barreales-Suárez et al., 2018). MS and MS/MS experiments were run in an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Xevo G2S QTOF mass spectrometer (Waters, Micromass, Manchester, UK) with electrospray ionization (ESI) and operating in both positive and negative ionization modes. A guard column Zorbax Eclipse XDB C-8 (12.5 mm × 2.1 mm i.d., 5 µm particle size) (Agilent Technologies Spain, Madrid, Spain) and a Zorbax Eclipse XDB-C18 analytical column (100 mm × 3.0 mm i.d., 3.5 µm particle size) settled at 25ºC were used to achieve the separation. Positive ionization separation was achieved using aqueous formic acid solution 0.05% (v/v) (solvent A) and acetonitrile (solvent B) with the following program: 90% A and 10% B increasing % B from 10% to 90% in 8.0 min, returning then to initial conditions in one minute

and waiting 3 minutes for re-equilibration of the column at a flow rate of 0.5 mL min^{-1}. Negative ionization elution followed the program: 70% A and 30% B initially, increasing the B percentage until 100% in 7.0 min and finally one minute more to returning to initial conditions in 2 min for re-equilibration time before next injection. The injection volume was 5 µL. Detection conditions used were as follows: nebulization gas 600 L/h, cone gas (nitrogen) 30 L/h, desolvation temperature 400 °C, source temperature 100 °C for positive polarity and 120 °C for negative polarity, capillary voltage 1.5 kV for positive ionization and −2.00 kV for negative, and sample cone voltages 40 V. MS/MS experiments were acquired employing argon (99.995%, Praxair) as collision gas and the collision energies and mass data are depicted in supplementary material (table S1). Chromatograms and spectra for each sample extracted were acquired and processed by MassLynx™ 4.1 software (Waters Corporation, Milford, MA, USA).. To quantitate the six pharmaceuticals, matrix matched calibration curves obtained by spiking blank extracts at ten concentration levels in the range 0.5 to 500 ng mL^{-1} was used. The peak area signals obtained from MS chromatograms for each compound (at its accurate mass) in the extracts from the samples were finally interpolated in the regression calibration curves. Detection limits (LODs) of the applied method were within 2.2-21 $ng \cdot g^{-1}$for *Salicornia europaea* and 4.6- 30 $ng \cdot g^{-1}$ for *Juncus sp*. and quantitation limits (LOQs) were within 7.2- 69 $ng \cdot g^{-1}$for *Salicornia europaea* and 15- 101 $ng \cdot g^{-1}$ for *Juncus sp*. (Barreales-Suárez et al., 2018)

3. Results and discussion

The uptake study described in section 2.2 was applied to plants of *Juncus sp.* and *Salicornia europaea* in order to evaluate somehow, by means of a laboratory experiment, the incidence that the presence of pharmaceuticals in waters that irrigates wetlands and natural areas might have. The assay was carried out using two botanical species usually present in aquatic ecosystems, which are endemic in southern Spain, where some protected nature reserves exist. Thus, the variety *Juncus sp.* is usually found in freshwater aquatic ecosystems while the variety *Salicornia europaea* is, very often, present in coastal ecosystems , such as marshes or coastal wetlands, located along the shores and river mouths.

As it is been reported before (Wu et al., 2013; Marsoni et al., 2014), the uptake of pharmaceuticals and their translocation (ratio of concentration in leaves over concentration in roots) through plant parts depends on the hydrophobic nature (logP) and ionization state of the molecules which would imply that they are dependent on the pKa of the compound, the capacity of ion exchange of the soil and the pH of the soil. This is explained because the bioaccumulation by roots is reduced as ion crosses membranes slower than neutral molecules, so the fraction of neutral molecule (f_n) (Trapp et al., 2009) a priori might suggest the ability of the compound to be absorb by the plant. The soils used in this study were pH 6.5 and 6.8 in the case of *Juncus sp.* and *Salicornia europaea*, respectively, and table 1 depicts the logP values (octanol-water partition coefficient), pKa and log Dow (apparent octanol water distribution partition coefficient) for the pharmaceuticals used in the uptake assays.

Compound	Structure	Therapeutic class	log P[a]	pKa[a]	(f_n)[b] (pH 6,5)	(f_n)[b] (pH 6,5)	log Dow[b] (pH 6,5)	log Dow[b] (pH 6,8)
CPR		Antibiotic	0.65	6.43 8.63	0.460	0.299	0.31	0.12
CBZ		Antiepileptic	2.45	13.9	1.00	1.00	2.45	2.45
DCL		Non steroidal antinflamatory	4.02	4.15	0.004	0.002	2.16	1.86
ENR		Antibiotic	1.88	6.43 7.75	0.460	0.299	1.54	1.35
FMQ		Antibiotic	2.41	5.70	0.137	0.074	1.55	1.27
IBU		Non steroidal antinflamatory	3.72	4.41	0.008	0.004	2.31	2.01

[a] Predicted values from database of Royal Society of Chemistry: http://www.chemspider.com.
[b] Calculated from Wu et al. 2013.

Table 1. Physicochemical properties of selected pharmaceuticals.

3.1. Uptake study in *Juncus sp.*

Regarding the appearance of the plant during the assay, at 14 days it was observed that the plants subjected to high concentrations started to acquire a slight yellow color, while the others did not experience any visually perceptible change. In the third week, the plants exposed to medium concentration of spiking solution, began to lose their characteristic bright green color, while plants exposed to high concentrations lost their rigidity, and, started to wilt, while those subjected to low concentrations, as well as the controls, did not experience any change. In the fourth and last week of the test the plants exposed to high and medium concentrations withered completely while the plants exposed to low concentrations started to show signs of evident deterioration, acquiring a dull green colour. The control plants, however, showed no change throughout the whole experiment.

All active ingredients were detected in *Juncus sp.* except IBU which could be attributed to the low f_n (<0.01), which suggest a high ionization of the molecule, and the weak hydrophobic character determined by its log Dow (table 1) (Wu et al., 2013; Hurtado et al., 2016; Montemurro et al., 2017). In general, the plants exposed to higher concentrations (10 $\mu g \cdot mL^{-1}$) had significantly higher levels of pharmaceuticals than those subjected to intermediate and low concentrations, showing, moreover, less variability in terms of concentrations found. Furthermore, similar behaviour was observed among the plants subjected to the same concentration levels in terms of the accumulation pattern over time.

As it can be observed in the results depicted in the table 2, CBZ, FMQ and CPR showed quantifiable levels in all stem/leaf samples at all exposure levels, which is in agreement with previous reported results (Wu et al., 2013; Riemenschneider et al., 2017b; Martinenez-Piernas et al., 2017). In the case of CBZ, at high exposure level, an increase in accumulation was observed up to 21 days, stabilizing in the last week of the assay, probably due to the fact that the plants were no longer able to accumulate and metabolize as much CBZ as the high values found which would corroborate the plant severe damage. CBZ have been reported to be highly bioavailable and have showed a great capacity for translocation within plants due to its weak basic character and pH independent value of log Dow (Tanoue el al., 2012) which would explain the high concentrations found. A completely different scenario was found for CBZ at concentrations of 700 and 10 $ng \cdot mL^{-1}$, where the maximum concentrations were measured at 14 days decreasing subsequently until the last week of assay, which

might mean that the plants would have been able to eliminate or metabolize the drugs before ending up dying at the end of the assay. At the root, CBZ records the highest values, reaching the order of 140,000 $ng \cdot g^{-1}$; this could be due to the fact that, as explained in the survey by Kodesova et al., 2019 CBZ would be absorbed through separate pathways, water uptake occurs mainly through regulated water channels or aquaporins, depending on the water demand of the plant, and the non-ionic CBZ molecule is translocated mainly by a diffusion mechanism through the root cell membranes, hence high amounts of CBZ were found in the plant roots (Migliore et al., 2003). However, some authors have reported data that showed a mainly accumulation on leaves instead of roots (Wu et al., 2014 and Montemurro et al., 2017).

In the case of FMQ, in contrast, at high exposure level, the concentration remained stable for three weeks, increasing greatly in the last week of the assay, which could be associated with the difficulty of this species to absorb this active ingredient despite the relative hydrophilicity of the compound as expressed by its log Dow values (table 1). Similar results were also observed in the accumulation profiles at medium and low concentration levels, as well as in the root which are in agreement with that obtained previously by Migliore et al., 2000. Lack of translocation from roots to the aboveground compartments at short exposure times was also describe by for norfloxacin, a fluoroquinolone very similar to FMQ.

Respecting CPR, plants subjected to a 10 $\mu g \cdot mL^{-1}$ irrigation solution showed a great accumulation of active principle in the first week, however, the values dropped sharply in the second week, to remain stable until the end of the assay. This could be due to the damages suffered by the plants during the whole uptake study, since as weeks passed, these plants showed an evident deterioration, which could make difficult the assimilation and metabolization of CPR. This behavior is completely different from that observed in plants subjected to medium and low exposure levels, where no quantifiable levels were observed in the first week and then increased to remain stable until the end of the assay. Nevertheless, plants subjected to low concentrations did not show values from which did not show significant accumulation, as most samples did not show quantifiable values. Some previous works have discussed the uptake and translocation of CPR in plants (Lillenberg et al., 2010, Migliore et al., 2003) which would agree in an extent with the results obtained in our work, but differs

266 with the given by other authors in which CPR showed very low or null levels of accumulation in the
267 foliage (Eggen et al., 2011, Sabourin et al., 2012).
268 Plants exposed to low level of concentration did not showed ENR. However, plants exposed to
269 concentrations of 10 $\mu g \cdot mL^{-1}$ showed a gradually increase on accumulation over the time, but this
270 increase was slight along the assay. Plants irrigated with 700 $ng \cdot mL^{-1}$ maintained, however a constant
271 accumulation trend until the end of the assay from the second week of the experiment. The levels
272 found in roots were lower than those found for FMQ or CBZ but in the same order of magnitude of
273 CPR. This situation agrees with data reported by Lillenberg et al., 2010 who reported concentrations
274 of ENR and CPR in leaves of lettuce (*Lactuca sativa*), common barley (*Hordeum vulgare L.*) and
275 cucumber (*Cucumis sativus L.*), however Hawker at al., 2013 did not find translocation for
276 norfloxacin in rice (which is similar in structure and physicochemical characteristics to ENR and
277 CPR). ENR and CPR are zwiterionics (table 1) at the pH values of the soils employed, and as it is
278 known, biological membranes have reduced permeability for zwiterions compared to neutral
279 molecules (Hawker at al., 2013) but according to (Panja et al. 2019) the 30% of CPR is translocated
280 from roots. Low levels found for ENR and CPR in root samples and at low and medium exposure
281 levels, might reflect two possible scenario, the ability of this plants to assimilate and eliminate, via
282 metabolization, these drugs, what it would be evidenced by the high concentrations found for CPR
283 at high level of exposure, or on the contrary, the difficulty of the plants to absorb the compounds.
284 In the case of DCL the accumulation patterns are completely different at the different level of
285 exposure, thus, concentrations reached a maximum in the second week of the assay to drop slightly
286 at 21 days and then suffering a huge increase at the end of the assay when the plants were subjected
287 to higher concentrations but, at medium level of exposure, a rising accumulation trend at 14 days and
288 then a stabilizing over the time was observed. A fast rate of translocation of DCL from roots have
289 been previously described by Bartha et al., 2014 in *Typha latifolia*, the authors reported that DCL
290 concentrations increased in leaves in just one day of exposure at 1 $\mu g \cdot mL^{-1}$ and then remained
291 constant until the end of the study (one week). A similar accumulation trend as described by Bartha
292 et al., 2014 was obtained in this work at medium level of exposure, this shows once again that the

293 mechanisms of assimilation, accumulation and detoxification of the plants depend largely on the
294 concentration to which the plants are exposed.
295 At the root, in general, higher concentrations were observed at higher levels of exposure. Despite
296 this, it was only possible to quantify CBZ and FMQ in irrigated plants at low concentration. Although
297 it was expected to find a correlation between the results of stem/leaf accumulation and the roots, this
298 was not reflected in some of the drugs analyzed. Thus, the high levels of concentration found in the
299 samples for CBZ, FMQ and DCL would suggest an apparent difficulty in the absorption of these
300 compounds to the plant, however, in general terms, the values in the stem/leaf samples analyzed
301 showed very high concentrations at the end of the assay for these compounds which would indicate
302 their easily absorption and distribution to the rest of the plant. In the case of CPR, despite the high
303 values found in the first week, and the great decrease in subsequent weeks, the values found in the
304 root are remarkably low. This could be due to the possibility that ENR is partially metabolized to
305 CPR (Migliore et al., 2003; Lillenberg et al., 2010) producing an initial increase in its concentration
306 value, which would later decrease with the progressive plant damage, and therefore the apparent
307 difficulty in its absorption, as described before.

Matrix	Level Plant	Cuts	Found compounds concentrations ($ng \cdot g^{-1}$)											
			CBZ R1		CBZ R2		FMQ R1		FMQ R2		CPR R1		CPR R2	
			$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD
Stem/leaf	10 $\mu g \cdot mL^{-1}$ P1/ P2	7 days	$2.41 \cdot 10^3$	509	$1.05 \cdot 10^3$	3	$1.96 \cdot 10^3$	12	924	22	$19.0 \cdot 10^3$	746	$19.4 \cdot 10^3$	58
		14 days	$8.08 \cdot 10^3$	306	$25.2 \cdot 10^3$	18	$1.95 \cdot 10^3$	60	$2.16 \cdot 10^3$	82	$1.80 \cdot 10^3$	50	$1.13 \cdot 10^3$	126
		21 days	$38.9 \cdot 10^3$	812	$39.0 \cdot 10^3$	827	$2.18 \cdot 10^3$	170	$2.28 \cdot 10^3$	185	$1.30 \cdot 10^3$	0	$1.37 \cdot 10^3$	8
		30 days	$39.9 \cdot 10^3$	384	$39.9 \cdot 10^3$	$1.5 \cdot 10^3$	$50.4 \cdot 10^3$	611	$53.2 \cdot 10^3$	376	$1.49 \cdot 10^3$	48	$1.14 \cdot 10^3$	11
														
	700 $ng \cdot mL^{-1}$ P3/ P4	7 days	----	----	----	----	----	----	----	----	----	----	----	----
		14 days	$7.03 \cdot 10^3$	118	$3.38 \cdot 10^3$	317	612	22	454	28	190	4	189	18
		21 days	$4.04 \cdot 10^3$	196	$2.41 \cdot 10^3$	48	543	38	420	39	205	27	177	14
		30 days	$1.28 \cdot 10^3$	55	$3.11 \cdot 10^3$	785	$2.90 \cdot 10^3$	70	$3.90 \cdot 10^3$	36	198	11	151	3
														
	10 $ng \cdot mL^{-1}$ P5/ P6	7 days	----	----	----	----	----	----	----	----	----	----	----	----
		14days	213	9	200	7	68	1	66	1	167	0.2	----	----
		21 days	89	25	204	1	140	1	138	2	160	10	----	----
		30 days	19	7	10	1	375	12	395	0.5	----	----	----	----
														
Root	10 $\mu g \cdot mL^{-1}$		$88.8 \cdot 10^3$	$5.35 \cdot 10^3$	$139 \cdot 10^3$	144	$41.4 \cdot 10^3$	$5.79 \cdot 10^3$	$51.4 \cdot 10^3$	50	900	132	640	8
	700 $ng \cdot mL^{-1}$		$42.6 \cdot 10^3$	$4.44 \cdot 10^3$	$29.0 \cdot 10^3$	$1.77 \cdot 10^3$	$7.17 \cdot 10^3$	$1.04 \cdot 10^3$	$4.61 \cdot 10^3$	196	----	----	----	----
	10 $ng \cdot mL^{-1}$		$1.43 \cdot 10^3$	41	$2.88 \cdot 10^3$	161	96	3	183	4	----	----	----	----

[a]Average concentration (n= 2)

Table 2. Concentrations ($ng \cdot g^{-1}$) obtained in *Juncus sp.* samples after uptake assay.

Matrix	Level Plant	Cuts	Found compounds concentrations ($ng \cdot g^{-1}$)							
			ENR [a] R1		ENR [a] R2		DCL+ [a] R1		DCL+ [a] R2	
			$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD
Stem/leaf	10 $\mu g \cdot mL^{-1}$ P1/ P2	7 days	627	10	566	1	$1.56 \cdot 10^3$	134	$1.07 \cdot 10^3$	98
		14 days	$1.45 \cdot 10^3$	329	$3.38 \cdot 10^3$	118	$2.47 \cdot 10^3$	131	$3.01 \cdot 10^3$	275
		21 days	$3.16 \cdot 10^3$	240	$3.25 \cdot 10^3$	255	$1.60 \cdot 10^3$	60	$1.66 \cdot 10^3$	68
		30 days	$3.99 \cdot 10^3$	11	$4.46 \cdot 10^3$	440	$24.0 \cdot 10^3$	555	$39.5 \cdot 10^3$	$1.41 \cdot 10^3$
										
	700 $ng \cdot mL^{-1}$ P3/ P4	7 days	---	---	---	---	---	---	---	---
		14 days	606	24	559	65	$1.43 \cdot 10^3$	14	536	125
		21 days	576	6	488	64	254	7	306	41
		30 days	662	26	590	9	377	20	632	107
										
	10 $ng \cdot mL^{-1}$ P5/ P6	7 days	---	---	---	---	---	---	---	---
		14 days	---	---	---	---	---	---	---	---
		21 days	---	---	---	---	---	---	---	---
		30 days	---	---	---	---	---	---	---	---
Root	10 $\mu g \cdot mL^{-1}$		$4.39 \cdot 10^3$	445	$3.52 \cdot 10^3$	44	$79.3 \cdot 10^3$	807	$127 \cdot 10^3$	$2.39 \cdot 10^3$
	700 $ng \cdot mL^{-1}$		508	106	----	----	$7.18 \cdot 10^3$	209	$3.53 \cdot 10^3$	150
	10 $ng \cdot mL^{-1}$		---	---	----	----	---	---	----	----

[a]Average concentration (n= 2)

Table 2 (cont.). Concentrations ($ng \cdot g^{-1}$) obtained in *Juncus sp.* samples after uptake assay.

308 3.2. Uptake study in *Salicornia europaea*

309 During the first week, plants exposed high concentrations experienced significant change, losing
310 rigidity in the stems and acquiring a grayish color, plants exposed to low and medium concentrations
311 started to lose the green and fleshy color in the stems. In the second week, plants exposed to high
312 concentrations worsened dramatically, all stems lost stiffness and almost the entire plant had a
313 shriveled appearance. However, plants exposed to medium and low concentrations did not change
314 from the previous week and controls remained unchanged. During the third week, the plants exposed
315 to high concentrations were practically dead, while the rest of the exposed plants suffered a
316 significant damage. Therefore, it was decided to end the uptake assay after 21 days, even though the
317 controls presented a healthy appearance.

318 Accumulation results were only obtained for the active ingredients CBZ and FMQ (table 3),
319 probably, and as mentioned above, because the uptake of the active ingredients highly depends on
320 their hydrophobic nature (log P), which determines their absorption by the plant, depending highly
321 on the species involved (Tanoue et al. 2012). Reproducible values have been registered in plants
322 irrigated at intermediate and high concentrations (700 $ng \cdot mL^{-1}$ and 10 $\mu g \cdot mL^{-1}$) where the plants
323 behaved similarly during the course of the study. However, at concentrations of 10 $ng \cdot mL^{-1}$ no
324 accumulation of any active substance was found during the assay. The concentration of the drugs did
325 not reach quantifiable levels, so it is likely that either the plant's absorption was low or the metabolism
326 was high, the former being more likely since no positive results were neither obtained in the root
327 samples.

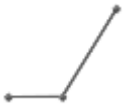
328 In general, the accumulation trends show the same rising evolution for both active ingredients, in
329 contrast to the results obtained in *Juncus sp.* assay where the behavior over the time could be
330 considered analyte-dependent, as already observed in our previous work on lavender plants with the
331 same compounds (Barreales-Suárez et al., 2018).

332 Results depicted in table 3 shows a clear increase in the CBZ accumulation in the last week of the
333 assay, at 21 days, at medium and high level of exposure, while in the first 14 days, a slightly rising
334 trend was observed, probably because the plant was not able to assimilate the CBZ until 14 days had
335 elapsed.

In the case of FMQ, we are facing a similar situation, with a high increase in the last week of the assay, which is less pronounced at 700 $ng \cdot mL^{-1}$ exposure level, although in this case, the concentrations measured in the second cut-off clearly exceed the accumulations of CBZ in the same period of time, indicating that *Salicornia europaea* was able to absorb and metabolize FMQ to a greater extent than CBZ.

In the root of the plant, only FMQ and CBZ were found, in samples exposed to medium and high concentrations, showing in the case of FMQ accumulations of the same order of magnitude as those obtained in the stem/leaf samples in the last week of the study. The levels of CBZ, however, were much lower, which would support the results found in stem/leaf samples, highlighting the difficulty of this to be absorbed by the plant.

The rest of analytes investigated were not found in any of the analyzed samples, which might reflect a situation analogous to that reported in a previous work where *Lavandula dentata* (Barreales-Suárez et al., 2018) in which, probably due to the low pKa values of these analytes, their absorption through the roots turns difficult in *Salicornia europaea.*

Matrix	Level Plant	Cuts	Found compounds concentrations ($ng \cdot g^{-1}$)							
			CBZ R1		CBZ R2		FMQ R1		FMQ R2	
			$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD	$\bar{x}$ [a]	SD
Stem/leaf	10 $\mu g \cdot g^{-1}$ P1/ P2	7 days	130	18	109	33	76	1.4	73	5
		14 days	221	1	241	5	$3.20 \cdot 10^3$	93	$2.71 \cdot 10^3$	54
		21 days	$23.5 \cdot 10^3$	112	$42.9 \cdot 10^3$	$3.38 \cdot 10^3$	$12.6 \cdot 10^3$	$1.35 \cdot 10^3$	$7.38 \cdot 10^3$	389
										
	700 $ng \cdot g^{-1}$ P3/ P4	7 days	22	1	23	4	61	0.4	----	----
		14 days	42	3	51	3	186	2	213	8
		21 days	$9.91 \cdot 10^3$	964	$7.81 \cdot 10^3$	134	567	6	354	81
										
	10 $ng \cdot g^{-1}$ P5/ P6	7 days	----	----	----	----	----	----	----	----
		14 days	----	----	----	----	----	----	----	----
		21 days	----	----	----	----	----	----	----	----
Root	10 $\mu g \cdot g^{-1}$		$9.11 \cdot 10^3$	633	$7.91 \cdot 10^3$	80	$13.4 \cdot 10^3$	$2.91 \cdot 10^3$	$11.7 \cdot 10^3$	912
	700 $ng \cdot g^{-1}$		437	20	172	20	280	88	287	67
	10 $ng \cdot g^{-1}$		----	----	----	----	----	----	----	----

[a]Average concentration (n= 2)

Table 3**.** Concentrations ($ng \cdot g^{-1}$) obtained in *Salicornia europaea* samples after uptake assay.

4. Conclusions

In the present work, an UPLC-QTOF/MS method was used for the quantitation of six pharmacological active substances in different parts (stem/leaf and roots) of *Juncus sp.* and *Salicornia europaea* subjected to an uptake study. The results showed that the accumulation of the active ingredients evaluated depend to a large extent on the type of plant, thus, in *Juncus sp.* most of the analytes were measured at quantifiable levels at almost all the exposure concentrations and very different behaviors were observed, not only among the active principles analyzed, but also among the three exposure levels to which the study was conducted.

However, in *Salicornia europaea* only two of the active ingredients used for this accumulation assay could be quantified. In addition, the results showed a quite different behavior to *Juncus sp.* plants, showing an accumulation in all parts of the plant analyzed over the time. This clearly reveals the need to carry out these studies in order to evaluate and identify the environmental effects that the discharge of emerging pollutants has on ecosystems, which go further than the damage that may be suffered by the plants, since, as shown in this study, these can accumulate significant levels of certain pharmacological active ingredients. Although several information on their toxicological effects is available there is still a lack of information about their presence, fate and effects in aquatic and terrestrial ecosystems, from which they could eventually enter into the trophic chain with serious consequences.

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Legends of tables.

Table 1. Physicochemical properties of selected pharmaceuticals.

Table 2. Concentrations ($ng \cdot g^{-1}$) obtained in *Juncus sp.* samples after uptake assay.

Table 3. Concentrations ($ng \cdot g^{-1}$) obtained in *Salicornia europaea* samples after uptake assay.

Supplementary Material

Table S1. Retention times (RT), full MS and MS/MS fragments ions elemental composition and collision energy for each analyte.

"Supplementary Material"

Compound	Elemental Composition	Monoisotopic mass	Precursor ion	extracted m/z (precursor)	RT (min)	Collision Energy (eV)	m/z Frag. Ions	Fragment ions
CPR	$C_{17}H_{18}FN_3O_3$	331.1332	$[M+H]^+$	332.1407	2.7	15	288.1501	$C_{16}H_{19}FN_3O$
							314.1303	$C_{17}H_{17}FN_3O_2$
							245.1084	$C_{14}H_{14}FN_2O$
							268.1430	$C_{16}H_{18}N_3O$
ENR	$C_{19}H_{22}FN_3O_4$	359.1645	$[M+H]^+$	360.1723	2.9	15	316.1728	$C_{18}H_{23}FN_3O$
							342.1618	$C_{19}H_{21}FN_3O_2$
							245.1090	$C_{14}H_{14}FN_2O$
CBZ	$C_{15}H_{12}N_2O$	236.0950	$[M+H]^+$	237.1024	5.0	20	194.0964	$C_{14}H_{12}N$
FLM	$C_{14}H_{12}FNO_3$	261.0801	$[M+H]^+$	262.0875	5.4	20	244.0776	$C_{14}H_{12}FNO_2$
							202.0305	$C_{11}H_5FNO_2$
							220.0410	$C_{11}H_7FNO_3$
DCL+	$C_{14}H_{13}Cl_2NO_2$	295.0167	$[M+H]^+$	296.0240	7.2	20	214.0418	$C_{10}H_6N_4O_2$
							250.0191	$C_{13}H_{12}Cl_2N$
							278.0135	$C_{14}H_{12}Cl_2NO$
IBU	$C_{13}H_{18}O_2$	206.1306	[M-H]-	205.1230	5.3	6	161.1336	$C_{11}H_{18}$
DCL-	$C_{14}H_{13}Cl_{12}NO_2$	295.0167	[M-H]-	294.0081	5.1	20	214.0418	$C_{10}H_6N_4O_2$
							215.0504	$C_{14}H_{13}ClNO$
							250.0191	$C_{13}H_{12}Cl_2N$
							278.0142	$C_{14}H_{12}Cl_{12}NO$

Table S1. Retention times (RT), full MS and MS/MS fragments ions elemental composition and collision energy for each analyte (mass error allowed <5 ppm).